ANTIBODIES AGAINST ANNEXINS, USE THEREOF FOR THERAPY AND DIAGNOSIS. USE OF ANNEXINS FOR THERAPY AND DIAGNOSIS.

## Description

The present invention relates to anti-annexin antibodies and their uses as well as uses of theirs ligands. Such annexins and anti-annexin antibodies are useful for detecting apoptosis and for the production of pharmaceutical compositions for the diagnosis and/or treatment of diseases linked to apoptosis, such as cancer, autoimmune diseases, cardiovascular and/or vascular diseases.

Apoptosis constitutes the most common form of cell death throughout the lifespan of an organism. During development and adulthood the removal of excess cells by apoptosis ensures tissue homeostasis.[1,2]

Upon apoptotic stimuli such as cytotoxic agents e.g. staurosporine, γ-irradiation or the engagement of the CD95 (APO-1/Fas) receptor, cell death proceeds via activation of caspases, finally leading to the formation of membrane enclosed apoptotic bodies.[3,4]

To preclude the release of noxious cellular contents, the remnants of dying cells are rapidly cleared by neighboring cells and phagocytes like macrophages or dendritic cells.[5,6] This efficient and safe uptake is mediated via specific signals or ligands, which must provide for engulfment as well as prevention of immune responses against self antigens.[7,8]

Accordingly, apoptotic cells have been reported to mediate anti-inflammatory signals to phagocytosing cells, thereby actively down-regulating the immune response.[9,10] Thus, in contrast to necrotic cell death, apoptosis causes little, if any, immune response.

Annexin I belongs to a well conserved family of lipid and calcium binding

proteins with tissue- and development-specific expression.[11] Expression of annexin I can be detected in a variety of tissues including all cells of hematopoietic origin except erythrocytes, neuronal cells and epithelial as well as endothelial cells.[12] In contrast to other members of the annexin family like annexin IV and V, annexin I expression is absent in cells of the hepato-pancreatic system in the adult organism. However, expression of annexin I in hepatocytes can be detected during early stages of embryogenesis. Moreover, annexin I expression can also be induced in adult hepatocytes after treatment with IL-6 and HGF and was reported to be strongly expressed in human hepatocellular carcinoma cells[13-15]. Therefore, expression of different members of the annexin family by a specific cell is well regulated and shows considerable plasticity. As determined so far, most if not all tissues express members of the annexin family to varying degrees. Notably, tissues in which expression of annexin I, II and IV cannot be detected show expression of annexin V instead, as for example astrocytes and skeletal muscle as well as cardiac muscle cells.[16,17]

Sharing a conserved lipid binding core, mammalian annexins differ considerably in their N-terminus.[11] While annexin V possesses an N-terminus of only 14 amino acids [18,19], the N-terminus of annexin I consists of 40 amino acids and contains several target sequences for phosphorylation.[20] In a number of studies, annexin I has been implicated in the suppression of inflammation. While originally described as an intracellular mediator of glucocorticoid actions,[21,22] an extracellular form of annexin I has been shown to regulate neutrophil extravasation through interaction with the formyl peptide receptor.[23] In addition, mice deficient for annexin I show increased and aberrant inflammation,[24] adding further evidence to a role for annexin I in the control of inflammation. Reported anti-inflammatory properties of annexin I have been linked to its unique N-terminus as well as to a sequence of its core domain, which shows homology to a class of anti inflammatory peptides, known as antiflammins.[25]

Neutrophils represent the largest entity of lymphocytes in the blood. Due to their short half-life, neutrophils also constitute the major load of apoptotic cells to be disposed of under physiological conditions.[17] Neutrophils also express high levels of endogenous annexin I,[18] which is translocated to the surface upon prolonged culture *in vitro*. Simultaneously, the N-terminus of annexin I is cleaved (Fig. 4).

Dendritic cells (DCs) are the most potent antigen presenting cells and their activation and maturation are critical for the outcome of an immune response.[19] Residing in an immature state in peripheral tissues, DCs can be activated upon encountering danger signals like the bacterial cell wall component lipopoly-saccharides (LPS).[20,21] Following activation, DCs mature and migrate towards lymphatic tissues. DC maturation induces the expression of costimulatory surface markers like MHC class II molecules and members of the B7 family as well as the secretion of pro-inflammatory cytokines like TNFα and IL-12.[22,23] Under steady state conditions, DCs have been shown to contain engulfed apoptotic material.[24] Under these conditions, molecules on the surface of these engulfed apoptotic cells might prevent subsequent activation of the respective DCs and, thus, preclude an autoimmune response.[25]

USP 5,658,877 discloses pharmaceutical compositions containing safe and effective amounts of annexin I and methods for treating lung disease by alleviating the effects of endotoxin.

USP 5,632,986 discloses a conjugate comprising an annexin covalently bound to urokinase. The preferred annexin is annexin V.

USP 6,312,694 discloses methods for killing tumor vascular endothelial cells by administering a binding ligand comprising a cytotoxic agent operatively linked to a targeting agent. One of the targeting agents mentioned is annexin V. There is no hint of a connection between annexin V and apoptosis.

US 5,296,467 discloses pharmaceutical compositions consisting essentially of a vascular anticoagulant annexin, $Ca^{2+}$ and $Zn^{2+}$.

Apoptosis is a mechanism which could be used to eliminate cells without the deleterious effects of necrosis. It would be highly desirable if this mechanism could be used for cancer therapy.

It was therefore an object of the present invention to provide substances which allow the detection and/or monitoring apoptosis.

This object is solved by providing an anti-annexin antibody or a fragment thereof which is capable of binding specifically to an annexin present on a cell that is undergoing apoptosis.

It was surprisingly found that an antibody generated to be specific for an annexin, in particular anti-annexin I and further anti-annexin II, anti-annexin IV and anti-annexin V antibodies as well as antibodies against other members of the annexin family, can be used to detect apoptosis and to diagnose, monitor and/or treat a variety of conditions including cancer.

Such an anti-annexin antibody or fragment thereof preferably binds to annexin I or annexin V. Further preferred antibodies of the present invention include anti-annexin I and further anti-annexin II, anti-annexin IV and anti-annexin V antibodies as well as antibodies against other members of the annexin family. Such antibodies can be used for example and without being limited thereto, for the detection of apoptosis *in vitro, ex vivo or in vivo*.

A preferred such antibody of the present invention is DAC5 or a fragment thereof, which is described in the experimental part.

In order to discover new molecules involved in the signaling of apoptotic cells towards phagocytes, mice were immunized with apoptotic Jurkat cells and monoclonal antibodies were generated. One of these antibodies, termed DAC5 for "Detector of Apoptotic Cells Nr.5", recognized a protein on the surface of early apoptotic but not live Jurkat cells (Fig 1a). Binding kinetics of DAC5 resembled the kinetics of the widely used early apoptosis marker annexin V. Notably, DAC5 binding occurred before membrane integrity of the cells was lost, as measured by propidium iodide (PI) uptake. This indicates that the antigen was localized on the cell surface. Binding was not restricted to the apoptotic stimulus nor to the Jurkat cell line, as binding occurred also after treatment with leucine zipper CD95 (LZ-CD95), which triggers the CD95 (APO-1/Fas) pathway, as well as upon γ-irradiation (Fig 1b). Cells binding DAC5 upon apoptosis included primary human T cells, HeLa cells and the melanoma cell line A375 (data not shown). Using immunoprecipitation of Jurkat lysates resolved on a silverstained SDS-PAGE and subsequent mass spectroscopy, the antigen of DAC5 was identified as annexin I (Fig 2).

It is shown here that upon induction of apoptosis, annexin I is translocated to the outer cell membrane, where it binds to negatively charged phospholipids in a calcium dependent fashion. Consequently, we could detect increasing amounts of annexins I, II and IV in EDTA membrane-washes of early apoptotic cells (Fig. 3).

Thus, the antibody of the present invention allows the detection and also the monitoring of all stages of apoptosis, including early stages.

To investigate the role of surface annexin I on apoptotic cells in the generation of immune responses, we tested the effects of annexin I positive d1 neutrophils on the maturation of dendritic cells (DC).

We therefore measured the TNFα release and DC surface markers after

coincubation of immature DCs with annexin I-positive d1 neutrophils and subsequent LPS incubation. As a control we also incubated late apoptotic d4 neutrophils with DCs, in which the full length annexin I is almost completely degraded (Fig 4). In contrast to d4 neutrophils, d1 neutrophils inhibited the LPS-induced maturation of DCs as measured by TNFα secretion and upregulation of the maturation markers CD83, CD86 and HLA-DR (Fig.5a). When surface annexin I was blocked by the addition of DAC 5 $F(ab)_2$-fragments, however, the suppression exerted by d1 neutrophils was remarkably released (Fig 5b).

Surprisingly, it was found that annexin I probably serves as an endogenous "anti-inflammation" signal on the surface of apoptotic cells. In the course of physiological tissue turnover, annexin I prevents the activation of antigen presenting cells that have engulfed apoptotic cells. Thereby, annexin I helps to maintain tolerance towards self antigens derived from apoptotic cells and might serve as a powerful tool to control immune responses during transplantation and autoimmunity. In addition, anti-annexin I antibodies such as DAC5 can be used to block the development of tolerance against tumors.

Being a marker specific for apoptotic cells, DAC5 might also facilitate the detection of apoptotic cells *in vivo*. To this aim, tumors were established in immune-compromised NOD/SCID mice, using apoptosis sensitive (J16) and apoptosis resistant (Rapo) Jurkat clones, respectively. Apoptosis in the sensitive tumor cells was induced through i.v. injection of the apoptosis inducing antibody anti APO-1.[26] 24 h after injection tumors were isolated and tested for apoptosis by flowcytometry. Indeed, the sensitive tumor cell showed 30% apoptosis, while while the resistant tumor showed only background apoptosis levels (Fig 6).

To monitor DAC5-binding to the sensitive tumor *in vivo*, the antibody was labeled with radioactive $^{131}I$ and tested for binding on apoptotic cells. As can be seen in Fig 7, $^{131}I$ DAC5 retained its capability to bind to apoptotic cells. To verify the potential of DAC5 to detect apoptotic cells *in vivo*, further studies are ongoing. In

addition, a recent report from Oh *et. al.* showed the presence of annexin I on the outer surface of endothelial cells lining the vessels in the vicinity of tumors. Tumor therapy using an anti-annexin I antibody was successful in reducing tumor burden and survival of tumor bearing rats.[27] Therefore, anti-annexin I antibodies might as well be suitable for targeting therapeutic agents directly to tumors, even if tumor cells themselves do not express annexin I.

The antibody of the invention is preferably a monoclonal antibody. The antibody of the invention can be a whole antibody, or it can be a fragment, such as a Fab or $F(ab')_2$ fragment.

The antibody or fragment according to a preferred embodiment of this invention is labeled. The label should enable detection *in vitro* or *in vivo* or *ex vivo*, preferably in such a way that a diagnosis *in vivo* can be performed. Labels which are suitable therefore are known to the man in the art as well as methods for detecting this label. Examples for such labels include radioactive labels, luminescent labels. An example is FITC.

The label can also be an effector molecule or a toxic substance, e.g. a cytotoxic effector molecule which is able to destroy cells, e.g. tumor cells. Examples are radioactive or toxic substances. Such substances can be very specifically directed to the tumor locus by using the antibody according to the present invention, and such antibodies bind to tumor cells which due to apoptosis show annexin I or other annexins on their surface. The effector substance which is coupled to the antibody can destroy not only the cell to which the antibody is coupled via annexin I, but also destroys cells in the environment of such apoptotic cells, which themselves are not yet apoptotic or do not surrender to apoptosis for whatever reason.

From above-described studies and the studies which are presented in the following examples, several applications and uses can be deduced for annexins

in general, especially annexin I and further also annexin II, IV and V, as well as anti-annexin antibodies, such as anti-annexin I and further anti-annexin II, anti-annexin IV and anti-annexin V antibodies as well as antibodies against other members of the annexin family.

As mentioned above, annexins are expressed on cells that are undergoing apoptosis. Such cells are often found in tissue affected by diseases such as cancer or tumor tissue, and tissue affected by autoimmune diseases such as rheumatoid arthritis, lupus erythematosus and multiple sclerosis, as well as diabetes and cardiovascular and vascular diseases.

Surprisingly it was found that anti-annexin antibodies can actually be used to modulate immune responses. The use of the antibody according to the present invention in therapy and especially in tumor therapy as well as in support of commonly used tumor therapies can be mediated by the following mechanism: Cells showing annexin I on their surface block inflammatory signals within the immune system. Therefore, such cells are not destroyed efficiently by the immune apparatus. When antibodies bind to the annexin I molecules, they block the anti-inflammatory signals that are presented to the immune effector cell. As a result, an inflammation can take place which itself supports the immune response in for example cancer therapy as well as support of an immune response against self-antigens. When such antibodies are brought in contact with cells or tissue containing cells that are undergoing apoptosis, the anti-annexin antibodies will bind to the annexins expressed on the apoptosing cells. This allows not only the use of such antibodies for closely monitoring and detecting apoptosis *in vivo, in vitro and ex vivo,* but also the specific targeting of such cells and/or tissues for diagnostic and/or therapeutic purposes. By way of such targeting, it is possible to modulate the immune system with regard to its responses to such cells. Since annexins expressed on apoptotic cells appear to function as anti-inflammatory signals, anti-annexin antibodies directed to such cells will block such anti-inflammatory signals and thus elicit and/or increase the

inflammatory response to the cells and also to the surrounding cells. This way, it is possible to use such anti-annexin antibodies to induce and/or increae an inflammatory response to tumor tissue.

In addition, it is also possible to use anti-annexin antibodies which are coupled to an effector molecule which can be a toxic substance or a radioactive substance or a marker. When a toxic or cytotoxic substance is used as the effector molecule, the anti-annexin antibody will be able to not only target apoptosing cells such as those present in tumor tissue, but also to target the surrounding cells and damage and/or kill them. Thus, such anti-annexin antibodies will be very effective as anti-tumor agents, or they can be used to support or complement existing tumor and cancer therapies.

Labelled anti-annexin antibodies are very useful as diagnostic agents. One possible application of such antibodies is in the diagnosis of cancer. After an initial conventional cancer therapy, such as chemotherapy or radiation therapy, labelled anti-annexin antibodies can be administered to a patient. Such antibodies will bind annexins present on or in association with cells that have started to undergo apoptosis. Since apoptosis is likely to increase after cancer therapy, the antibodies will be able to detect tumor tissue and even micrometastases and other tumor cells which are otherwise very difficult to detect. Thus, the antibodies will allow a more specific detection and thus a directed and specific treatment of tumor cells and tumor tissue. In addition, anti-annexin antibodies could then be used to target those tumor cells and tumor tissues containing apoptosing cells to delete or damage the tumor cells and tissues by using antibodies coupled to effector molecules. Thus, such antibodies can be used to supplement and/or improve existing tumor therapies.

For some purposes the antibodies need not necessarily be labeled, whereas for other therapeutic or diagnostic methods it is preferred to use a labeled antibody. This label can be a molecule which allows for detection of the bound antibodies

*in vitro* or *in vivo* or a substance that is able to destroy cells, like for example radioactivity or a toxic substance. As mentioned above, such substances, which might be coupled to the antibodies according to the present invention are known to a person skilled in the art.

The same mechanism can also be used to treat and/or diagnose other diseases such as autoimmune diseases, which are also associated with cells undergoing apoptosis. Such diseases include, but are not limited to rheumatoid arthritis, lupus erythematosus, multiple sclerosis and diabetes and many other autoimmune diseases. In rheumatoid arthritis for example, it is possible to use anti-annexin antibodies to specifically target synovial cells and induce an inflammatory response to such cells and thereby delete them more efficiently. Other diseases than can be diagnosed and/or treated include, but are not limited to vascular and cardiovascular diseases

Because the anti-annexins function as anti-inflammatory signals, they might also be involved in the development of tolerance against tumor cells and other cells undergoing apoptosis. Thus, anti-annexin antibodies as described above can also be used for blocking the development of tolerance against tumor cells and/or tumor tissue.

Therefore, the use of the present antibody for treating tumors, either alone or in combination with common tumor therapies, may lead to complete eradication of cancer cells with no remaining resistance. In such treatment the apoptotic cells showing annexin I on their surface are bound specifically and destroyed by the effector agent, the cells surrounding such apoptotic cells, however, are also destroyed due to the effect of the effector molecule. The effector molecules can be selected according to their efficiency and their range of effectiveness.

Furthermore, the presence of annexin I on the vessel walls of tumor tissue might target anti-annexin I antibodies directly to tumors, even if the tumors do not

express annexin I. Accordingly, anti-annexin I antibodies and/or anti-annexin II, anti-annexin IV and anti-annexin V antibodies as well as antibodies against other members of the annexin family might be useful in therapy of any vascularized tumor, including small cell lung carcinoma.

This might be very important in cancer therapy, for example for monitoring the progress of an anti-tumoral therapy and further detection whether a tumor is amenable to such therapy. Malignancies whose therapy could be monitored using anti-annexin I antibodies include all annexin I expressing tumors like T cell lymphoma, Melanoma and tumors of epithelial and endothelial origin and neuronal tumors.[12,15] In addition, antibodies against annexins II, IV and V could be used to monitor malignancies that express the respective annexins, including tumors of the hepato-pancreatic system, of the intestinal tract and of muscle tissues.[12]

In the field of cancer therapy, the use of the present antibody can also support and/or complement a cancer therapy. Common tumor therapies often are able to destroy the majority of tumor cells, but for largely unknown reasons some cells remain resistant to tumor therapy and cannot be destroyed. Such cells might be the cause for further tumor growth after the first therapy has been terminated. The use of the present antibody for tumor therapy can prevent such later growth of tumors from resistant tumor cells.

Also, such antibodies can be used for a diagnosis of diseases with concomitant increased apoptosis rate of cells, as for example diabetes and auto-immune diseases. Especially preferred is the use of the anti-annexin I antibody for the detection of apoptosis *in vivo* and for the monitoring of therapy.

Such diseases include those mentioned above, including cancer but also diseases such as diabetes, autoimmune diseases and also cardiovascular and vascular diseases, such as those mentioned above.

Further, the invention relates to a method of detecting apoptosis comprising: (i) providing a sample to be analysed, comprising cells; (ii) detecting an annexin present on the surface of said cells by adding a substance capable of specifically binding to an annexin present on a cell that is undergoing apoptosis. Preferably, the substance of step (ii) is an antibody or fragment thereof capable of specifically binding to an annexin present on a cell that is undergoing apoptosis. The annexin to be detected is preferably annexin I, annexin II, annexin IV or annexin V, in particular full-length annexin I having a molecular weight of about 38 kDa. Thus, the antibody of fragment thereof is preferably specific for annexin I, in particular the 38 kDa annexin I (see Fig. 4).

Another subject matter of the present invention is the use of annexin I and/or further annexin II, annexin IV and annexin V as well as other members of the annexin family for certain indications. From the studies connected to the present invention, it could be gathered that annexin I, when presented on the cell surface, gives an anti-inflammation signal. Such anti-inflammation signals also occur when foreign tissues are transplanted into a patient. The use of annexin I can avoid such problem when administered to a patient or to tissue to be transplanted. Presentation of annexin I on such tissues suppresses the immune response that otherwise might be initiated due to immune recognition of the transplanted tissue.

Thus, administration of annexin I II, IV or V or fragments thereof leads to a marking of such transplantation tissue that avoids an inflammatory effect.

This effect obviously is true also for other members of the annexin family, wherefore use and/or application of other annexins to transplantation patients or

tissue to be transplanted is encompassed by the present invention, especially annexin I, II, IV or V, as another means according to the present invention to avoid inflammatory immune reaction against the transplanted tissues.

A further possibility of using the results of the present invention is that annexin I and other annexins are related to specific receptors, which again can be stimulated or blocked by either binding of one of the annexins or fragments thereof or an antibody against this receptor. Such binding of receptors by an annexin or an antibody against the annexin-receptor can also influence the immune response and lead to the same possibilities for therapy as described above for the annexin antibodies or the use of annexins in transplantation.

Thus, annexins and/or functional fragments thereof and/or fusion proteins comprising an annexin or functional fragments thereof can be used to modulate the immune system. When annexin proteins are isolated or recombinantly produced and contacted with cells and/or tissues containing cells undergoing apoptosis, they will bind to the surface of apoptotic cells. In some cases where there is increased cell death, the naturally occurring levels of annexin may not be sufficient to inhibit or suppress an inflammatory response, and thus the administration of annexins is useful for the prevention or reduction of such inflammation, thereby exerting a therapeutic or beneficial effect. Thus, they can be used to produce the same signals that annexins expressed by the apoptotic cells themselves will elicit. Thus, contacting cells or tissues with annexin proteins will lead to the production of anti-inflammatory signals. This can be used for the diagnosis and/or treatment of diseases which are linked with cell death in general, apoptosis and/or inflammation. Thus, annexins can be used to block inflammation or an inflammatory response. Diseases which are linked to inflammation and/or cell death include, but are not limited to ischaemia-reperfusion damage, stroke, chronic heart failure, myocardial infarction, spinal cord injury, acute liver failure, renal ischaemia, neurodegenerative diseases such as Alzheimer and Parkinson's disease, sepsis and HIV-infection. In the

same way, the annexins could be used to inhibit inflammatory responses to tissues such as transplantation tissues and the like. Other diseases that can also be treated by the administration of annexins or functional fragments thereof are autoimmune diseases such as multiple sclerosis, lupus erythenatosus, rheumatoid arthritis, diabetes and others.

In the acute phases of multiple sclerosis, large numbers of neurons and other cells of the nervous system, e.g. oligo dendrocytes, undergo apoptosis. Often, this massive cell death is accompanied by inflammation. The treatment of patients of MS, before, after or during such an acute phase, will suppress this inflammation and thus reduce the damage to the tissue.

The following example and figures are meant to further elucidate the present invention.

**Description of the Figures**

**FIGURE 1: DAC5 binds to the surface of apoptotic cells**

**a)**

Jurkat T cells were induced to undergo apoptosis by addition of 1 µM staurosporine (sts). At different time points, $2x10^5$ cells per sample were incubated with 100µl DAC5 supernatant or 1 µg/ml IgG2a isotype control Ab (iso), followed by incubation with FITC-labelled anti-mouse IgG antibodies. To discriminate between early and late apoptosis, aliquots were incubated in parallel with FITC-labelled annexin V (AxV) or 2 µg/ml propidium iodide (PI). The kinetic is representative for 3 experiments. Inset shows a representative DAC5 staining after 4 hours incubation with staurosporine (shaded histogram, in comparison to DAC5 staining on live cells.

**b)**

Jurkat T cells were irradiated with 150 Gy and cultured for additional 6 hours. Alternatively, cells were incubated for 6 hours with 1 µM staurosporine (sts) or

1 µg/ml leucine zipper CD95 ligand (LZ-CD95L). Subsequently, cells were stained with FITC-labeled annexin V or FITC-labeled DAC5 in annexin Binding buffer.

**FIGURE 2: DAC5 precipitates annexin I**

**a)**

Postnuclear lysates of $10^7$ CEM cells were immmunoprecipitated with Protein A sepharose and purified DAC5 or IgG2a isotype control (iso), or protein A sepharose alone (-). Precipitates were resolved on a 12% SDS PAGE and silver stained. The protein bands labeled "annexin I" were cut from the gel and subjected to tryptic digest, followed by analysis via mass spectroscopy. By comparison with the NCBInr database peptides were identified as derived from annexin I. hc – heavy chain; lc – light chain.

**FIGURE 3: annexin I is bound to the outside of apoptotic cells**

$5x10^6$ CEM cells were treated with 1 µM sts for different time points and centrifuged to obtain the supernatant. The pellets were washed with PBS/EDTA to yield the membrane bound annexin I (membrane). Finally, cell pellets were lysed and aliquots of all fractions were resolved on a 12% SDS PAGE followed by detection with monoclonal anti-annexin I Abs. Western Blot shown is representative for 3 experiments.

**FIGURE 4: annexin I on the surface of cultured neutrophils**

Primary human neutrophils were cultured for 1-4 days. Aliquots were analyzed for annexin I and phosphatidylserine-exposure by flowcytometry, using annexin V-FITC or DAC5-FITC, respectively. annexin I protein levels were monitored on Westernblot, using DAC5 and a peroxidase coupled, secondary anti mouse IgG antibody.

**FIGURE 5: annexin I suppresses DC cytokine secretion**

$2x10^5$ d7 immature DCs were incubated for 4h with or without 50µg/ml DAC5-F

$(ab')^2$-Fragments and $1x10^6$ d1 neutrophils. As controls, d1 neutrophils were added in a transwell chamber and d4 neutrophils were used, respectively. Following 4h of coculture, 50ng/ml LPS were added to the wells for another 48h. Cells were then harvested, stained with antibodies against CD83, CD86 and HLA-DR and analyzed by flowcytometry (a). Aliquots of cell culture supernatants were collected 24 h after addition of LPS and analyzed with ELISAs for TNFα (b).

**FIGURE 6: Jurkat tumor model**

$5x10^6$ apoptosis sensitive Jurkat J16 cells or apoptosis resistant Rapo cells were injected s.c. into NOD/SCID-mice. When tumors of ca. 8 mm in diameter were established, the mice received an injection of 250 µg anti-APO-1. After 24 h the tumors were explanted. Cells were separated and analyzed for apoptosis using flowcytometric analysis of DNA content after Nicoletti *et al* (ref).

**FIGURE 7: Iodinated DAC5 binds to apoptotic cells *in vitro***

$1x10^6$ CEM cells were irradiated with $200mJ/cm^2$ UV-C light and cultured for another 4h. Apoptotic cells were then incubated with $^{131}$I-DAC5 or $^{127}$I-DAC5 for 20 min at 4°C. Cells incubated with the radioactively labeled DAC5 were centrifuged through oil and analyzed for bound radioactivity in a beta-counter. Cells stained with $^{127}$I- DAC5 were analyzed by flowcytometry, using a secondary FITC-coupled anti mouse IgG antibody.

**EXAMPLES**

**Example 1**

***Immunization and fusion***

C57BL/6 mice were kept under certified pathogen free conditions and immunized nine times i.p. with $2x\ 10^7$ apoptotic J16 Jurkat T cells. Apoptosis was induced by incubation with 1 µM staurosporine (Sigma, Munich, Germany) for 0.5 h. Cells were thoroughly washed in sterile PBS before injection. During the

first 3 immunizations, the apoptotic J16 cells were coated with 1,2 µg/ml recombinant human annexin V (Sigma, Munich, Germany) to promote generation of high affinity antibodies[28]. Hybridomas were generated from spleens of immunized mice by Polyethylene glycol-induced fusion with the mouse plasmacytoma line Ag8.

**Example 2**

***Cell lines and hybridomas***

The human T-ALL cell line CEM and the human Jurkat T cell lines J16 and Rapo were cultured in RPMI 1640 (Gibco, Karlsruhe, Germany) supplemented with 10% fetal calf serum (Gibco, Karlsruhe, Germany) and 10 mM HEPES (Sigma, Munich, Germany). Supernatant of the hybridoma DAC 5 was affinity purified using a Protein A–Sepharose column (Sigma, Munich, Germany), followed by dialysis against PBS. $F(ab')_2$ fragments of DAC5 were generated using Immunopure kits (Pierce, Rockford, USA) according to manufacturers instructions.

**Example 3**

***Preparation of primary human dendritic cells (DC)***

Human immature DCs were prepared from PBMC of 500 ml heparinized blood of healthy donors. PBMC were prepared as described previously[29] using density centrifugation on a Ficoll gradient (Biochrom, Berlin, Germany) and allowed to adhere to plastic tissue culture flasks for 1h. Subsequently, adherent monocytes were washed thoroughly with PBS and seeded into 6 well cell culture plates at a density of $3x10^6$/well and cultured in X-Vivo 15 medium (Cambrex, Verviers, Belgium) supplemented with 2% inactivated donor serum and containing 1000 U/ml hu GM-CSF and 500 U/ml hu IL-4 (Immunotools, Osnabrück, Germany). Fresh cytokines were supplemented again on day 3. Immature DCs were used on day 7, and >98% were positive for the DC marker CD11c.

**Example 4**

***Preparation of primary human neutrophils***

Human neutrophils were obtained as described previously,[30] using density centrifugation of heparinized whole in PolymorphPrep (Axis Shield, Oslo, Norway). After preparation, neutrophils were cultured in X-Vivo 15 Medium (Cambrex, Verviers, Belgium), supplemented with 2% donor serum for up to 4 days (d1-d4 neutrophils).

**Example 5**

***Immunoprecipitation and silver staining***

For immunoprecipitation, $1x10^7$ J16 cells were lysed in Triton X-100 Lysisbuffer (20 mM Tris/HCl, pH 7.4, 1 % Triton X-100, 10 % glycerol, 150 mM NaCl, 1 mM PMSF and 1 µg/ml of Leupeptin, Antipain, Chymostatin and Pepstatin A) for 15 min on ice and centrifuged (15 min, 14000xg, 4°C). Supernatants were then subjected to immunoprecipitation with 10 µg DAC5 or isotype control antibody and Protein A Sepharose (Sigma, Munich, Germany) at 4°C overnight. Precipitates were prepared in SDS sample buffer, resolved on a 12% SDS PAGE and stained with the Silverquest silver staining kit (Invitrogen, Paisley, UK), according to manufacturers instructions.

**Example 6**

***Protein Sequencing***

Protein bands were excised from the silver stained gel, repeatedly washed with water, 40 mM ammonium bicarbonate/ ethanol (1:1 v/v), reduced with 10 mM DTT at 56 °C for 1 h and alkylated with 55 mM iodoacetamide at 25°C for 30 min in the dark. After alkylation, gel bands were repeatedly washed with 40mM ammonium bicarbonate and ethanol, dehydrated with 100% acetonitrile and air-dried for 15 min. Digestion with trypsin (10ng/µl, Promega) was performed in 40 mM ammonium bicarbonate at 37°C overnight.

**Example 7**

***MALDI-TOF mass spectrometry (MALDI-TOF-MS)***

Sample preparation was achieved by cocrystallization of ZipTip C18 (Millipore) concentrated samples with matrix. Peptides in the supernatant of the in-gel digest were absorbed to a prewashed (50% acetonitrile/water) and equilibrated (0.1% trifluoroacetic acid/water) ZipTip C18 by repetitive pipetting steps. Following washing of the ZipTip C18 by equilibration buffer the peptides were eluted from the ZipTip with 1 µl of matrix (α-cyano-4-hydroxycinnamic acid saturated in 50% acetonitrile/water). MALDI-TOF mass spectra were recorded in the positive ion reflector mode with delayed extraction on a Reflex II time-of-flight instrument (Bruker-Daltonik). Ion acceleration was set to 26.5 kV, the reflector was set to 30.0 kV and the first extraction plate was set to 20.6 kV. Mass spectra were obtained by averaging 50 to 200 individual laser shots. Calibration of the spectra was performed internally by a two-point linear fit using the autolysis products of trypsin at m/z 842.50 and m/z 2211.10. Post source decay (PSD) analysis was performed in the positive ion reflector mode with delayed extraction by setting an ion gate width of 40 Da around the ion of interest. Data were acquired in 14 segments by decreasing the reflector voltage in a stepwise fashion. For each segment 100 to 200 individual laser shots were accumulated. The fragment ion spectrum was obtained by pasting together all segments to a single spectrum using the FAST software provided by Bruker. Fragment ion calibration was performed externally with the fragment masses of the adrenocorticotropic hormone 18 - 39 clip.

**Example 8**

***Database search***

Singly charged monoisotopic peptide masses were used as inputs for database searching. Searches were performed against the NCBInr database using the ProFound search algorithm (http://129.85.19.192/prowl-cgi/ProFound.exe). Isoelectric points were allowed to range from 0 to 14, carbamidomethylation was included as fixed modification and the oxidation of methionine was allowed as possible modification. Up to one missed tryptic

cleavage was considered, and the mass tolerance for the monoisotopic peptide masses was set to +/-100 ppm. Searches with fragment masses from PSD experiments were performed against the NCBInr database using the MS-Tag search algorithm provided by the Protein Prospector software package (http://prospector.ucsf.edu). Parent mass tolerance was set to +/- 0.1 Da and fragment ion tolerance was set to +/- 0.9 Da).

**Example 9**

***Surface staining***

5 x $10^5$ cells were incubated with 100 µl of hybridoma culture supernatant or with 10 µg/ml purified DAC5 in PBS/10% FCS for 30 min at 4°C, washed with 500 µl PBS/10% FCS, and incubated another 30 min with 5 µg/ml FITC-labeled goat anti mouse IgG antibodies (Dianova, Hamburg, Germany) and 2 µg/ml propidium iodide (Sigma, Munich, Germany) in PBS/10% FCS. The addition of 10% FCS provided for calcium, necessary for annexin I to bind to the membrane of apoptotic cells. After further washing, cells were analyzed on a FACScan cytometer (Becton Dickinson, San Jose, USA). Staining with annexin V-FITC (Molecular Probes, Leiden, Netherlands) was performed according to manufactures instructions in annexin Binding buffer (10 mM HEPES, 140 mM NaCl, 2,5 mM $CaCl_2$, pH 7,4).

**Example 10**

***EDTA-washes and Western Blot analysis***

5x$10^6$ CEM cells were incubated in 500 µl medium with 1 µM staurosporine for different time periods or left untreated. Subsequently, the cells were centrifuged, the supernatant was collected and pellets were washed with 500µl PBS/10mM EDTA. After centrifugation the washing solution was collected and the pellets were lysed in 500 µl Triton X-100 Lysis buffer. A 200 µl aliquot of each fraction was separated on a 12 % SDS-PAGE, blotted onto nitrocellulose membrane (Amersham, Freiburg, Germany) and detected with monoclonal antibodies against annexin I (Pharmingen, San Diego, USA).

**Example 11**

***Cocultures of immature DCs and neutrophils***

Immature d6 DCs were plated at a density of $2x10^5$ cells/well into 24-well plates and incubated over night. The next day, d1 neutrophils were incubated for 15 min at 37° with 50µg/ml DAC5 $F(ab')_2$ fragments or left untreated, and overlaid onto the DCs at a ratio of 1:1. After 4 hours, 50 ng/ml LPS (Sigma, Munich, Germany; E.coli strain 026:B6) was added to a part of the cocultures. As controls, 50 µg/ml DAC5 $F(ab')_2$ fragments alone and d4 neutrophils were cocultured with DCs as well. After 48h the cells were harvested, centrifuged and stained with antibodies against HLA-DR, CD86 and CD83 (Caltag) and in PBS containing 10% mouse serum to block Fc-Receptor interactions. Cells were analyzed on a FACScan cytometer (Becton Dickinson, San Jose, USA). Supernatants were collected after 24 h and stored at - 20 °C for further cytokine analysis with ELISAs for TNFα (Pharmingen, San Diego, USA).

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